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Population genetics of the Mauritian flying fox, *Pteropus niger*

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The Mauritius flying fox *Pteropus niger* is distributed on the islands of Mauritius and La Réunion in the western Indian Ocean. Although recent studies have examined the phylogenetics and systematics of this genus, relatively few have assessed the population genetics of species distributed on oceanic islands and no study has focused on the demographics of *P. niger*. Here, we present mitochondrial DNA sequence data from 39 individuals of *P. niger* collected from four main colonies distributed throughout Mauritius. Our results indicate that the Mauritian population of *P. niger* is likely panmictic, with moderate to high levels of gene flow occurring among colonies distributed across the island. Collectively, our sequence data suggest moderate levels of genetic variation within the population. These findings will help to inform ongoing conservation and disease surveillance initiatives.

Key words: genetic structure, Mauritius, phylogeography, *Pteropus niger*

INTRODUCTION

In part associated with their wing structure and size, nocturnal fruit bats of the Old World family Pteropodidae are notably strong flyers with respect to other chiropterans (Norberg and Rayner, 1987). Non-forest dependent species are able to cross considerable distances in search of food resources and while dispersing. For example, some members of the genus *Pteropus*, which readily cross non-forested areas, are able to fly over 50 km in a given night (Palmer *et al.*, 2000; Tideman and Nelson, 2004). This capacity to cross substantial areas, including open water, has allowed members of this genus to colonize notably remote islands across the Old World tropics. Surprisingly, even though these bats can disperse across considerable oceanic areas, they nonetheless exhibit considerable levels of endemicity on islands and island groups, including those in the Indian Ocean. Hence, patterns of dispersal are by no means regular and different types of filters exist that provide mechanisms for local speciation (Chan *et al.*, 2011; Almeida *et al.*, 2014).

The Mauritian flying fox *Pteropus niger* is restricted in its distribution to the Mascarene Islands (Mauritius and La Réunion) in the southwestern Indian Ocean. On the basis of subfossil evidence, it is known that this species was once widespread on La Réunion, but was locally exterminated in the early 18th-century, almost certainly associated with local hunting pressure (Bergmans, 1999; Cheke and Hume, 2008). In 2007, a small colony of *P. niger* was identified on La Réunion, probably representing a recent recolonization (Roué and Probst, 2010; Hutson and Racey, 2013) and attesting to the ability of this species to disperse the ≈180 km of open water between these two islands. On Mauritius, which has a surface area of 1,860 km^2, this species remains widespread and relatively common. The local population was estimated in 2010 as 49,000–56,000 individuals, and thus considered stable (Sookhareea, 2011; Hutson and Racey, 2013). Recent phylogenetic work on the genus *Pteropus* indicates that *P. niger* diverged less than 500,000 years ago as part of a western Indian Ocean radiation (Almeida *et al.*, 2014) and can be considered a relatively recent taxon.

In the context of ongoing zoonoses study of *P. niger* on Mauritius, as well as understanding aspects of this species' population structure associated with conservation actions, it was deemed important to obtain molecular genetic data to examine patterns of gene flow among populations and to generally assess levels of genetic diversity. Prior to this current study, DNA sequence data had been generated from only five individuals of *P. niger* (O'Brien *et al.*, 2009; Almeida *et al.*, 2014) and all genetic analyses of the species have focused entirely on the systematics and phylogeography of the genus, and in some cases, with special reference to relationships among species distributed throughout the western Indian Ocean (O'Brien *et al.*, 2009; Chan *et al.*, 2011; Almeida *et al.*, 2014). The limited distribution of *P. niger* coupled with habitat loss of native forest on Mauritius and hunting pressure associated with consumption of commercially important fruit crops (Nyhagen, 2004; Nyhagen *et al.*, 2005; Price, 2013) has contributed to the classification of the species as vulnerable to extinction (Hutson and Racey, 2013). The Mauritian Government has not conducted to date any culling of *P. niger* (Florens, 2012) even given pressure from local fruit growers and exporters for this action to be taken. These different concerns underline the importance of understanding aspects of the population genetics of this species, which is the focus of this current study.

Our analyses utilize genetic variation in the mitochondrial control region (D-loop), a marker that has been frequently used to examine the genetic variation within natural populations of bats, including species of *Pteropus* (Salgueiro *et al*., 2004; Russell *et al*., 2008; Fleming *et al*., 2009; Brown *et al.*, 2011; Guevara-Chumacero *et al*., 2013). In addition to D-loop DNA sequence information, we generated sequence data from the mitochondrial cytochrome c oxidase subunit I (COI) gene from representative samples. DNA sequence data of the COI gene is frequently used for DNA barcoding initiatives, and prior to this study, no COI data had been available from *P. niger*. Collectively, the genetic data presented herein provides important insight into the population structure of this species across an island landscape that is about 45 km in width and 65 km in length. The patterns of genetic variation determined herein will contribute to ongoing conservation efforts, and will serve to inform future initiatives concerning the monitoring of emerging infectious diseases on Mauritius.

Materials and Methods

Sample Collection

With authorization from the Mauritian Government and in collaboration with National Parks and Conservation Service officers and their designated hunters, several known day roost sites of *Pteropus niger* were visited and a limited number of samples per site were collected (Fig. 1). All roosts were sampled in November of 2012 (Appendix). In the field immediately after capture, the animals were measured, prepared as standard museum fluid preserved specimens, and pectoral muscle samples saved in lysis buffer. Juveniles clearly associated with their mothers were only included in genetic analyses if sequence data was unavailable from the mother (Appendix). Voucher specimens are preserved and deposited in the Field Museum of Natural History, Chicago. For all specimens, organ tissue samples were conserved in liquid nitrogen for zoonoses studies. Research involving live animals followed the guidelines for the capture and handling of mammals approved by the American Society of Mammalogists (Gannon *et al.*, 2007).

Molecular Methods

Genomic DNA was extracted from muscle tissues using the DNeasy Blood and Tissue Kit (Qiagen Inc., Chatsworth, CA, USA). The mitochondrial D-loop and COI genes were amplified using primers reported in Brown *et al.* (2011). We used the

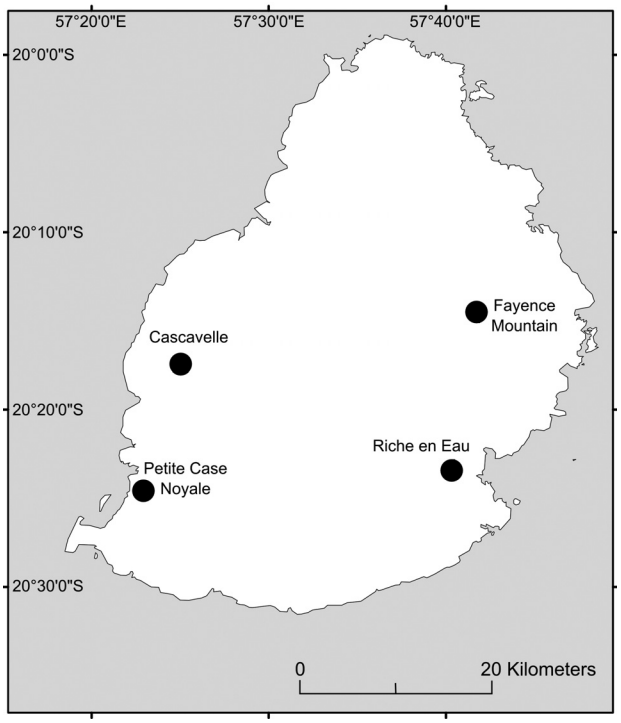

FIG. 1. Map of Mauritius showing the four main localities from which specimens of *P. niger* were collected. Genetic analyses were performed on 39 individuals (Cascavelle: $n = 16$, Petite Case Noyale: $n = 4$, Riche en Eau: $n = 6$, Fayence Mountain: $n = 13$)

Phusion High-Fidelity PCR Kit (New England Biolabs, Ipswich, MA, USA) for all PCR amplifications, which were performed with the following thermal profile: 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 50°C for 30 sec, and extension at 72°C for 45 sec with a final extension of 72°C for 10 min. PCR products were purified using the QIAquick PCR Purification kit (Qiagen Inc., Chatsworth, CA, USA). DNA sequencing was performed using ABI Big Dye version 3.1 chemistry and fragments were electrophoresed on an ABI 3700 Genetic Analyzer at the Duke Center for Genomic and Computational Genome Sequencing facility. Sequences were verified, assembled, and aligned using the Geneious software package (version 7.1) and the MAFFT alignment plugin (version 7.017). All sequence data reported herein was deposited in GenBank under the following accession numbers KP404015–KP404061.

Genetic Diversity and Demographic Analyses

MEGA (version 6.0 — Tamura *et al.*, 2013) software was used to determine the best-fitting model of DNA substitution for our D-loop sequence data. Arlequin software (version 3.5) was used to perform an analysis of molecular variance (AMOVA — Excoffier *et al.*, 1992) on the aligned D-loop sequence data in order to examine patterns of genetic variation within and among individuals. Between population variance was measured using the φ_{ST} statistic as implemented within Arlequin. Briefly, the φ_{ST} statistic provides a measure of the overall genetic variation within a population and values ranging from 0 to 0.05 are indicative of low genetic differentiation (frequent gene flow), whereas values greater than 0.25 are considered to represent strong genetic differentiation (very limited or no gene flow). Intermediate values of φ_{ST} (between 0.05 and 0.25) suggest moderate levels of genetic variability within the examined population. Unique D-loop haplotypes were identified using the CD-HIT clustering web server (www.cdhit.org) and a minimum spanning tree of depicting haplotype structure was generated using Arlequin. Nucleotide statistics were computed using DnaSP (version 5.0 — Librado and Rozas, 2009) and MEGA software packages and were compared to similar statistics generated from other species of *Pteropus* (Brown *et al.*, 2011). The demographic history of *P. niger* was examined using a mismatch distribution analysis (of all sampled individuals) as implemented in Arlequin. Briefly, populations constant in size typically show ragged mismatch distributions whereas those experiencing demographic expansion are unimodal (Rogers and Harpending, 1992). We implemented parametric bootstrapping in Arelquin (1,000 iterations) to test the goodness of fit of the observed mismatch distribution to that expected under a sudden expansion model using the sum of squared deviations statistic (SSD) and Harpending's raggedness index (*H* — Harpending 1994). Finally, we conducted a Mantel test (30,000 permutations) using the isolation by distance web server (www.ibdws.sdsu.edu) to examine the correlation between genetic and geographic distance among the four main colonies that were sampled.

RESULTS

We generated mitochondrial D-loop sequence data (383 base pairs) from 39 individuals of *P. niger* collected from four main localities across Mauritius (Fig. 1). Of the 39 sequences, 20 haplotypes were observed and several haplotypes were shared among the four collecting localities (Tables 1 and 2, Figs. 2 and 3). Average pairwise nucleotide diversity of the 39 D-loop sequences was 0.02 and 47 polymorphic base pairs were observed across the entire D-loop alignment (Table 1). The Tamura 3-parameter model of evolution was identified as the best fitting model for our data and was implemented in Arlequin. The AMOVA identified 0.05% of the overall genetic variation as occurring among the four populations (*d.f.* = 3, sum of squares [*SS*] = 14.49, variance = 0.00), while 99.95% of the genetic variation occurred within populations (*d.f.* = 35, *SS* = 168.41, variance = 4.81). The overall pairwise φ_{ST} statistic was 0.00 and this value was not significant ($P = 0.43$) (Table 3). The global mismatch distribution was multimodal (Fig. 4) and did not show significant departure from an equilibrium model (*SSD* = 0.011, $P = 0.26$) and Harpending's raggedness index was not significant ($H = 0.019$, $P = 0.21$). The Mantel test identified a negative correlation between genetic distance and geographic distance ($r = -0.53$) and this result was not statstically significant ($P = 0.87$). Analyses of 484 bp of the COI gene from nine individuals resulted in the identification of a single shared haplotype (zero DNA substitutions).

DISCUSSION

Our analyses indicate the absence of significant genetic structuring across the sampled population of *Pteropus niger* on Mauritius. This result is consistent with the ecology and behavior of *P. niger* (i.e. individuals are highly vagile and frequently visit a variety of roost sites on the island; see Hutson and Racey, 2013) as well as with other genetic analyses of insular pteropid bats (Brown *et al.*, 2011). Indeed, our mtDNA sequence data suggest enhanced gene

TABLE 1. Nucleotide statistics for 383 bp of mitochondrial control region DNA sequence from 39 individuals of *P. niger* collected from four main colonies on Mauritius (see Fig. 1). *n* = number of individuals sampled, *h* = number of haplotypes, *Hd* = haplotype diversity, *k* = average number of nucleotide differences, π = nucleotide diversity, *S* = number of segregating sites

Colony	*n*	*h*	*Hd*	*k*	π	*S*
Cascavelle	16	12	0.97	8.78	0.02	36
Petite Case Noyale	4	4	1.00	8.50	0.02	15
Riche en Eau	6	6	0.95	7.81	0.02	20
Fayence	13	9	0.94	11.10	0.03	36
Overall	39	20	0.95	9.39	0.02	47

TABLE 2. Between colony nucleotide variation across 383 base pairs of the mitochondrial control region from 39 individuals of *P. niger* (see Fig. 1); k — average number of nucleotide differences, π — nucleotide diversity

Colony comparison	Polymorphic nucleotides	π	Shared mutations	k
Cascavelle vs. Petite Case Noyale	37	0.02	14	8.50
Cascavelle vs. Riche en Eau	37	0.02	19	8.30
Cascavelle vs. Fayence	47	0.03	24	10.04
Petite Case Noyale vs. Riche	21	0.02	14	7.85
Petite Case Noyale vs. Fayence	36	0.03	14	10.35
Riche en Eau vs. Fayence	37	0.03	18	10.09

flow across the Mauritian population of this species (φ_{ST} = 0.00; Table 3) and multiple individuals of *P. niger* share identical mtDNA haplotypes at each of the four main collecting localities (Figs. 3 and 4). We also observed closely related mtDNA haplotypes among individuals collected from colonies separated by approximately 30 km (Fayence Mountain and Riche en Eau vs. Cascavelle and Petite Case Noyale; Figs. 1–4) and we identified a negative correlation between genetic and geographic distance (although not statistically significant). This result is consistent with the observation that the maximum geographic distances separating colonies of Mauritian *P. niger* are well within the average potential flight distances for members of this genus (Palmer *et al.*, 2000; Tidemann and Nelson, 2004; Breed *et al.*, 2010).

Collectively, our results indicate that the Mauritian population of *P. niger* exhibits moderate genetic variation when compared to other species of insular bats (overall haplotype diversity = 0.95; Fleming *et al.*, 2009) and that the population has been relatively stable over time (Fig. 4). Despite this observation, *P. niger* is considered vulnerable due to ongoing habitat destruction and susceptibility to cyclone devastation (Hutson and Racey, 2013). The absence of significant geographic barriers to dispersal on Mauritius as well as the long-distance flight capabilities of members of this genus (Breed *et al.*, 2010; Roberts *et al.*, 2012), is consistent with the genetic data presented herein. Gene flow is likely occurring among the colonies of *P. niger* distributed throughout Mauritius; however, this gene flow may occur with slightly less frequency among colonies separated by greater geographic distances (e.g., Cascavelle vs. Fayence Mountain and Riche en Eau — Table 3). Although we did not identify significant correlations between genetic and geographic

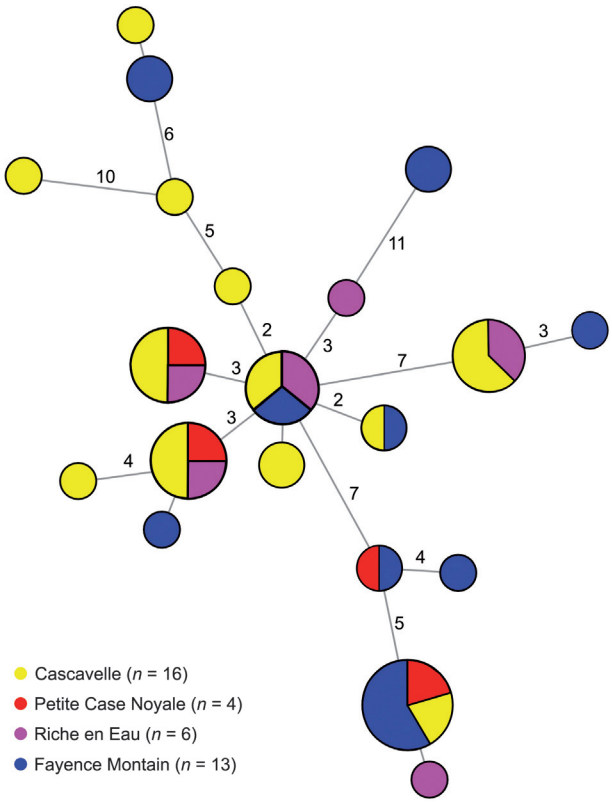

FIG. 2. Minimum spanning tree of *P. niger* mitochondrial control region haplotypes. Thirty-nine individuals were sampled and 20 mtDNA haplotypes were identified. Colors correspond to the four main collecting localities. Each circle represents a unique DNA sequence (haplotype) and the size of the circles represents the relative number of individuals having the same haplotype (smallest circles represent one individual). Relative frequencies of haplotypes shared among the four colonies are depicted as pie diagrams

TABLE 3. Pairwise φ_{ST} values (below diagonal) and Tamura 3-parameter genetic distances (above diagonal) between colonies of *P. niger*. Bold values show average Tamura-Nei genetic distance values within each location. The overall φ_{ST} value for the population was 0.00. Analyses were based on 383 bp of D-loop

Colony	n	1	2	3	4
Cascavelle	16	**0.023**	0.023	0.021	0.028
Petite Case Noyale	4	0.009	**0.023**	0.020	0.026
Riche en Eau	6	-0.059	0.041	**0.021**	0.026
Fayence	13	-0.097	-0.065	0.007	**0.030**

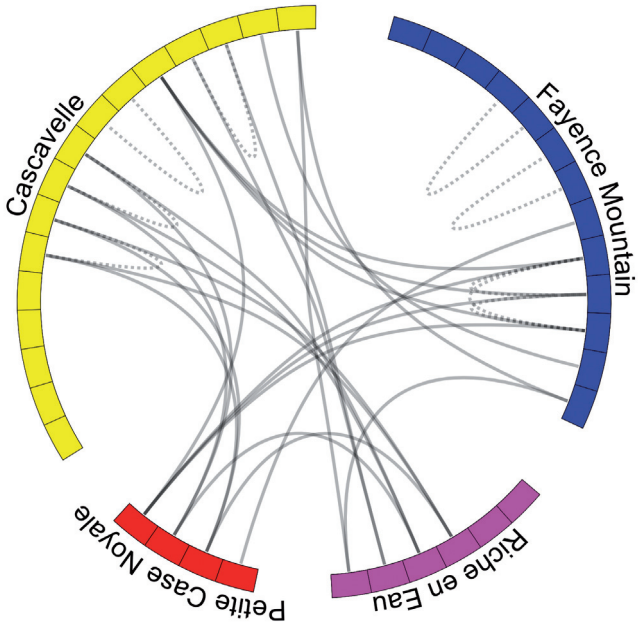

FIG. 3. Circos diagram showing shared D-loop haplotypes within and among the four collecting localities. For each location, colored segments represent individuals of *P. niger*. Dotted lines and solid lines depict shared haplotypes within and among localities, respectively. Segments without connecting lines are unique haplotypes

distances across the Mauritian population of *P. niger*, future conservation efforts may benefit from the management of geographically distant colonies. An obvious limitation with the present study is that it based on a sex-specific marker and could benefit from the addition of nuclear microsatellite data.

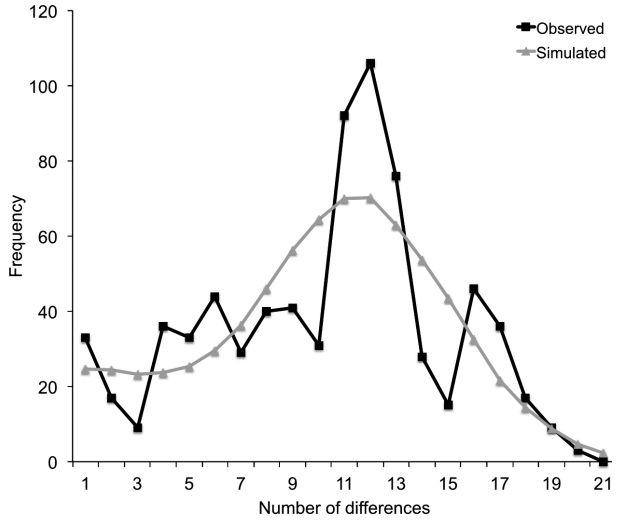

FIG. 4. Observed and expected mismatch distributions for *P. niger* from Mauritius. Black squares show observed distribution and gray triangles show theoretical expected distribution under the population expansion model

Inclusion of such data will help to better understand the population dynamics of *P. niger* and will allow for testing the hypotheses presented herein. Nevertheless, our mtDNA sequence data provide important insight into the genetics of *P. niger* as well as a greater understanding of the biology of this species.

Several species of *Pteropus* have been identified as reservoirs for emerging infectious diseases (e.g., Henipaviruses and Paramyxoviruses; Epstein *et al.*, 200 8; Halpin *et al.*, 2011; Drexler *et al.*, 2012; Hahn *et al.*, 2014). Antibodies to Nipah, Hendra, and Tioman viruses of the Paramyxovidae family have been detected in pteropodid bats from Madagascar (Iehlé *et al.*, 2007). Ongoing PCR based studies conducted on *P. niger* (sampling reported herein) has also detected paramyxovirus sequences; they, however, belong to the unclassified morbillivirus-related viruses (UMRVs) (Y. Gomard, unpublished data). UMRVs have already been reported in many small mammals on islands in the western Indian Ocean (Wilkinson *et al.*, 2014) and no pathogenic effect on humans has been reported to date.

Hence, the panmictic nature of the *Pteropus* population combined with increased interaction with the resident human population may pose some level of risk. Forthcoming work will focus on surveillance of infectious agents to elucidate potential risks associated with *Pteropus* spp. (Y. Gomard, unpublished data). The immediate concern should focus on the conservation of the Mauritian *P. niger* population, as the ongoing destruction of suitable habitat coupled with possible culling for commercial farming interests and climate change will likely have a negative impact on the *P. niger* population. Indeed, current estimates predict a 30% reduction of the *P. niger* population within the next ca. 20 years (www. iucnredlist.org).

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Appendix

List of specimens examined, including voucher numbers, GenBank accession numbers, geographic collecting locality, sex, and date of collection. All voucher specimens were deposited in the Field Museum of Natural History

Museum no.	Field no.	D-loop accession no.	COI accession no.	Collecting locality	Geographic coordinates		Sex	Date of collection
FMNH 221237	SMG 18056	KP404015	KP404054	Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♀	26-Nov-12
FMNH 221238	SMG 18057	KP404016	KP404055	Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♀	26-Nov-12
FMNH 221239	SMG 18058	KP404017	KP404056	Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♀	26-Nov-12
FMNH 221240	SMG 18059	KP404018		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♂	26-Nov-12
FMNH 221241	SMG 18060	KP404019		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♀	26-Nov-12
FMNH 221243	SMG 18062	KP404020		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♂ juv	26-Nov-12
FMNH 221244	SMG 18063	KP404021		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♀	26-Nov-12
FMNH 221246	SMG 18065	KP404022		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	? juv	26-Nov-12
FMNH 221247	SMG 18066	KP404023		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♀	26-Nov-12
FMNH 221248	SMG 18067	KP404024		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♀	26-Nov-12
FMNH 221250	SMG 18069	KP404025	KP404057	Black River District, Petite Case Noyale	20.40083°S	57.38332°E	♂	26-Nov-12
FMNH 221251	SMG 18070	KP404026	KP404058	Black River District, Petite Case Noyale	20.40083°S	57.38332°E	♀	26-Nov-12
FMNH 221253	SMG 18072	KP404027		Black River District, Petite Case Noyale	20.40083°S	57.38332°E	♀	26-Nov-12
FMNH 221254	SMG 18073	KP404028		Black River District, Petite Case Noyale	20.40083°S	57.38332°E	♂	26-Nov-12
FMNH 221255	SMG 18074	KP404029		Grand Port District, Riche en Eau	20.38925°S	57.67780°E	♂	27-Nov-12
FMNH 221256	SMG 18075	KP404030		Grand Port District, Riche en Eau	20.38925°S	57.67780°E	♀	27-Nov-12
FMNH 221257	SMG 18076	KP404031		Grand Port District, Riche en Eau	20.38925°S	57.67780°E	♂	27-Nov-12
FMNH 221258	SMG 18077	KP404032		Grand Port District, Riche en Eau	20.38925°S	57.67780°E	♀	27-Nov-12
FMNH 221259	SMG 18078	KP404033		Grand Port District, Riche en Eau	20.38925°S	57.67780°E	♂	27-Nov-12
FMNH 221260	SMG 18079	KP404034		Grand Port District, Riche en Eau	20.38925°S	57.67780°E	♀	27-Nov-12
FMNH 221262	SMG 18081	KP404035	KP404059	Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♀	28-Nov-12
FMNH 221263	SMG 18082	KP404036	KP404060	Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♀	28-Nov-12
FMNH 221264	SMG 18083	KP404037	KP404061	Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♀	28-Nov-12
FMNH 221266	SMG 18085	KP404038		Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♂ juv	28-Nov-12
FMNH 221267	SMG 18086	KP404039		Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♂	28-Nov-12
FMNH 221268	SMG 18087	KP404040		Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♀	28-Nov-12
FMNH 221269	SMG 18088	KP404041		Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♀	28-Nov-12
FMNH 221270	SMG 18089	KP404042		Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♂	28-Nov-12
FMNH 221271	SMG 18090	KP404043		Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♀	28-Nov-12
FMNH 221273	SMG 18092	KP404044		Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♀	28-Nov-12
FMNH 221274	SMG 18093	KP404045		Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♂	28-Nov-12
FMNH 221275	SMG 18094	KP404046		Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♀	28-Nov-12
FMNH 221278	SMG 18097	KP404047		Flacq District, northern slope Fayence Mountain	20.24350°S	57.70172°E	♂ juv	28-Nov-12
FMNH 221279	SMG 18098	KP404048		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♂	29-Nov-12
FMNH 221280	SMG 18099	KP404049		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♂	29-Nov-12
FMNH 221281	SMG 18100	KP404050		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♂	29-Nov-12
FMNH 221282	SMG 18101	KP404051		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♂	29-Nov-12
FMNH 221283	SMG 18102	KP404052		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♀	29-Nov-12
FMNH 221284	SMG 18103	KP404053		Black River District, Cascavelle, along Rivière Papayes	20.29532°S	57.41922°E	♂	29-Nov-12